FEBS Letters 584 (2010) 537–542

FEBS Letters

journal homepage: www.FEBSLetters.org

GLUT4 molecules are recruited at random for insertion within the plasma membrane upon insulin stimulation

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ARTICLE INFO

Article history:
Received 15 September 2009
Revised 30 November 2009
Accepted 30 November 2009
Available online 4 December 2009

Edited by Daniela Ruffell

Keywords:
Glucose transporter type 4
Insulin-responsive glucose transporter
Protein translocation
Protein trafficking
Insulin

ABSTRACT

Glucose transporter 4 (GLUT4) is efficiently retained intracellularly. Here, we investigated the insulin-induced reduction of retention. While increasing insulin concentrations led to gradual increases in both the amount of recycling GLUT4 molecules and cell surface GLUT4 levels, the kinetics of the increase in time was independent of insulin concentration. To determine whether there are GLUT4 subpools that have a distinct insulin sensitivity, adipocytes were consecutively stimulated twice with a low concentration of insulin while recycling GLUT4 molecules were continuously labeled. This revealed that not the same pool of GLUT4 molecules was mobilized twice and thus that upon insulin stimulation, GLUT4 is likely to be recruited at random for insertion within the plasma membrane.

1. Introduction

There are over a dozen facilitative sugar transporters in mammals. Glucose transporter 4 (GLUT4) plays an important role in glucose transport during the postprandial state and during exercise. This transporter is highly expressed in cell types that exhibit regulated glucose uptake, such as adipocytes, skeletal muscle cells, and cardiomyocytes. The intracellular traffic of GLUT4 is a major determinant of its acute regulation [1–3]. In the basal non-stimulated state, GLUT4 is present in a so-called GLUT4 storage compartment (GSC) from which it undergoes insulin-dependent movement to the cell surface and/or in GLUT4 storage vesicles (GSVs) that are released upon insulin stimulation. While the molecular entity of GLUT4 was revealed 20 years ago, we are only beginning to learn how its intracellular traffic and localization are organized. Recently, considerable progress in our understanding has been made due to the development and use of novel techniques and approaches [4–6]. However, how GLUT4 is retained intracellularly (i.e., refrained from being inserted into the plasma membrane) remains a mystery as is the process by which insulin signaling acts on this retention mechanism and provokes GLUT4 molecules to be released from their storage site.

Here, using a quantitative 96-well plate-based approach, we have investigated the intracellular storage of GLUT4 and the release of GLUT4 from its storage site upon insulin stimulation. Increasing insulin concentrations gradually increased both cell surface recycling and cell surface levels of GLUT4 without changing the kinetics of these intracellular trafficking steps, indicating that insulin dose dependently mobilizes a pool of GLUT4 molecules whose size is dependent on insulin concentration and that this pool largely determines cell surface GLUT4 levels. Importantly, our studies demonstrate that GLUT4 molecules are recruited by insulin at random and thus that these molecules are equal regarding their insulin sensitivity.

2. Materials and methods

2.1. Materials

3T3-L1 murine preadipocytes were obtained from the American Type Culture Collection ATCC/LGC Standards (Teddington, UK), bovine sera from PAA (Pasching, Austria), media and HEPES from Invitrogen (Carlsbad, CA), insulin from Lilly (Suresnes, France), monoclonal anti-HA antibody from Covance (Emeryville, CA), fluorescent antibodies from Molecular Probes/Invitrogen

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; GLUT4, insulin-responsive glucose transporter 4; GSC, GLUT4 storage compartment; GSV, GLUT4 storage vesicle; HA, hemagglutinin

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.11.093

(Carlsbad, CA), and black clear-bottom well plates from Greiner Bio-One (Kremsmunster, Austria). pBABE vector was kindly provided by Dr. Hartmut Land (University of Rochester, Rochester, NY).

2.2. Cell culture

The cDNA encoding HA–GLUT4 (GLUT4 with a hemagglutinin (HA) epitope tag in its first luminal domain) inserted in pBABE-puro vector is described elsewhere [7]. As the 3T3-L1 preadipocytes originally obtained from ATCC appeared to be very heterologous and differentiated very poorly, an individual clone of cells was isolated from these preadipocytes and tested for growth, differentiation capacity, and insulin-induced GLUT4 translocation. Cultures of this clone contained at least 95% adipocytes upon differentiation.

3T3-L1 preadipocytes were infected with retrovirus, cultured and differentiated as described before [7]. Adipocytes were used for experiments 8–12 days after onset of differentiation.

For insulin washout experiments, the adipocytes were stimulated with insulin as indicated, upon which the cells were washed twice with KRM buffer (20 mM MES pH 6.0, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.2% bovine serum albumin (BSA)), incubated for 1 min with KRM, for another 2 min with fresh KRM, washed with serum-free Dulbecco's modified Eagle's medium (DMEM), supplemented with 20 mM HEPES pH 7.4 and 0.2% BSA, and incubated for 50 min in DMEM/HEPES/BSA. All washes and incubations were performed at 37 °C.

2.3. Fluorescence-based techniques

The fluorescence-based assay for the detection of relative amounts of GLUT4 at the cell surface has been described in detail elsewhere [7]. Briefly, 3T3-L1 adipocytes expressing a GLUT4 molecule with an HA epitope tag in its first luminal domain were grown in 96-well plates. Adipocytes were incubated in the absence or presence of insulin, fixed in 3% paraformaldehyde, and immunolabeled using anti-HA antibody after the cells had been incubated in the absence or presence of the permeabilizing agent saponin to label HA–GLUT4 at the plasma membrane or total cellular HA–GLUT4, respectively. Cell surface GLUT4 levels were established by expressing the amount of HA–GLUT4 at the plasma membrane as percentage of total cellular HA–GLUT4. For antibody capture experiments, living adipocytes were incubated with saturating amounts of anti-HA antibody (50 μg/ml; purified by Protein G immunoglobulin purification kit; Pierce/Thermo Scientific; Rockford, IL), followed by extensive washes with ice-cold PBS and fixation. After incubation of the cells with fluorescent secondary antibody, fluorescence was measured using a microtiter plate reader (FLUOstar Galaxy, BMG Labtechnologies; Offenburg, Germany). Background values (basal condition) for cell surface GLUT4 levels and antibody capture were subtracted from those of insulin stimulations and compared to a maximal 100 nM insulin stimulation. Normal (non-immune) murine serum was used to determine non-specific antibody binding. Maximal increases in cell surface HA–GLUT4 and antibody capture in response to 100 nM insulin were 3-fold and 3- to 4-fold, respectively. After fluorescence measurements, plates were analyzed on a widefield fluorescence microscope (20× objective) to ensure that the signal was specific. All data are presented as means ± SE. Experiments were repeated at least four times. Representative experiments are shown. At least four wells were used for each data point.

For microscopy, preadipocytes were cultured on gelatin-coated coverslips and differentiated as usual. Adipocytes were incubated for two hours with 0.3 nM insulin in the presence of anti-HA antibody, followed by a mild acid treatment to remove insulin and to return the cells to basal state (see above), and a 1 h incubation in the absence or presence of 0.3 nM or 100 nM insulin. Control cells were incubated for two hours with anti-HA antibody in the absence or presence of 100 nM insulin. Upon fixation, the cells were immunolabeled with Alexa647-conjugated goat-anti-mouse antibody to stain anti-HA at the cell surface, permeabilized with saponin, and incubated with Alexa488-conjugated goat-anti-mouse antibody to label intracellular anti-HA. Cells were analyzed using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Göttingen, Germany) in the C3M MicorBio Cell Imaging Facility. The same PMT gain values were applied to all coverslips. These values were determined using the 2 h anti-HA/100 nM insulin incubation (i.e., pixels with maximal signal, without being saturated). Images were exported in pseudo-colours using the LSM 510 software.

3. Results and discussion

To gain information on how GLUT4 molecules are recruited upon insulin stimulation, we performed a study in which cell surface recycling and cell surface levels of GLUT4 were investigated in 3T3-L1 adipocytes that expressed ectopic GLUT4 with an HA tag in its first extracellular domain. This modified GLUT4 molecule behaves in many ways as endogenous GLUT4 as demonstrated by various research groups and is currently widely used in studies that deal with intracellular GLUT4 traffic [8–10].

First, we performed experiments in which living HA–GLUT4-expressing adipocytes were incubated with anti-HA tag antibody. In these experiments, GLUT4 molecules that recycle via the plasma membrane are instantaneously labeled with antibody and the amount of antibody captured by the cells therefore represents the pool of GLUT4 molecules that actively cycles via the plasma membrane. Thus far, this approach has been applied by a dozen laboratories and has proven to be a valuable tool in GLUT4 research. Several studies have demonstrated that the antibody correctly marks GLUT4 without disturbing its intracellular traffic. First, HA–GLUT4-bound anti-HA antibody has been shown to traverse the same organelles as wild-type GLUT4 [11]. Second, we have shown that even at endosomal pH, the anti-HA antibody does not dissociate from the HA tag within GLUT4 [5]. Third, antibody-bound GLUT4 is still readily responsive to insulin (Fig. 3B) [12]. Finally, culturing adipocytes for up to several days after a 1 h anti-HA antibody incubation has revealed that the degradation of the antibody closely resembles that of GLUT4 (i.e., 50 h half-life; data not shown) [13,14].

Previously, using this approach, we have demonstrated that only two-thirds of the total cellular GLUT4 pool can maximally be engaged in cell surface cycling, even under supraphysiologic insulin concentrations [5]. Incubating these adipocytes for two hours with increasing concentrations of insulin in the presence of saturating amounts of anti-HA tag antibody demonstrated that maximum and half-maximum amounts of GLUT4 could be labeled in the presence of 35 nM and 0.3 nM insulin, respectively (Fig. 1A), though the half-maximum dose varied significantly between individual experiments (e.g., Fig. 1A versus Fig. 1B versus Fig. 3CI). This likely represented the variation between cell cultures and appeared to be independent of cell passage and adipocyte age (i.e., time after initiation of differentiation). In agreement with our previous study [5], analysis of antibody capture in time demonstrated that the amount of recycling GLUT4 molecules at steady state augmented with increasing insulin levels (Fig. 1B). Here, by expressing antibody capture as percentage of maximum level for each concentration of insulin, we demonstrate that the time course of antibody capture was independent of insulin concentration, that the time it took to label the GLUT4 molecules to equilibrium was independent of insulin concentration, and that insulin merely

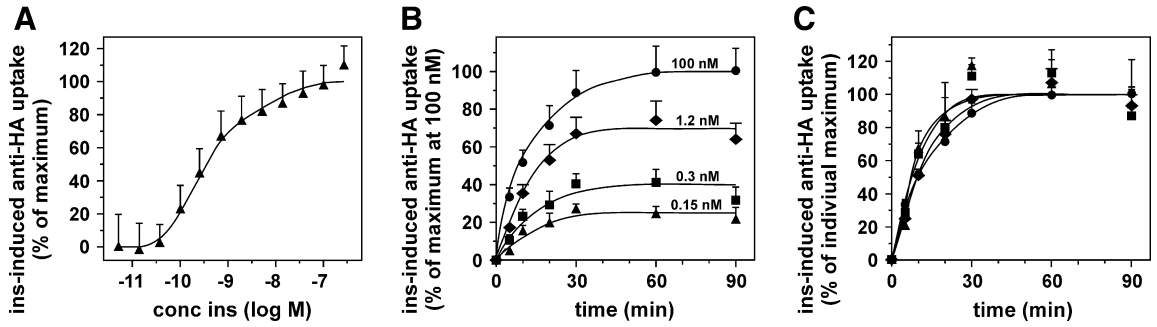


Fig. 1. Dose-dependent insulin-induced increases in the amount of GLUT4 that participates in cell surface recycling. (A) 3T3-L1 adipocytes expressing HA-tagged GLUT4 were incubated for 2 h with various concentrations of insulin in the presence of a saturating concentration of anti-HA antibody. The amount of antibody captured by the cells was determined and expressed as percentage of the maximum amount of captured antibody. (B) Adipocytes were incubated for various times with the indicated concentrations of insulin in the presence of anti-HA antibody and the relative amounts of antibody captured by the cells was determined and expressed as percentage of the maximum amount of capture possible in response to insulin. (C) Data from (B) are expressed as percentage of maximal response for each individual insulin concentration.

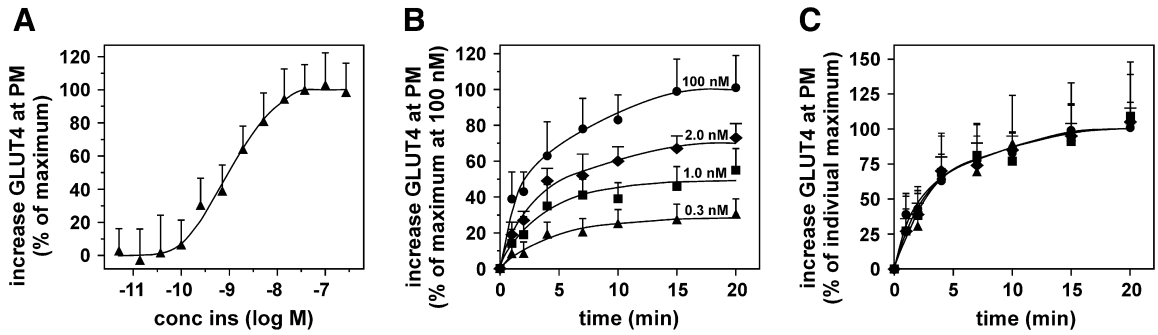


Fig. 2. Dose-dependent insulin-induced increases in cell surface GLUT4 levels. (A) Adipocytes were incubated for 20 min with various concentrations of insulin and relative cell surface GLUT4 levels were determined as described in Section 2 and expressed as percentage of maximum increase in response to insulin. (B) Adipocytes were incubated for various times with the indicated concentrations of insulin and relative cell surface GLUT4 levels were determined and expressed as percentage of maximum possible increase in response to insulin. At 20 min, steady state levels were reached and longer incubations did not result in higher cell surface GLUT4 levels. (C) Data from (B) are expressed as percentage of maximal response for each individual insulin concentration.

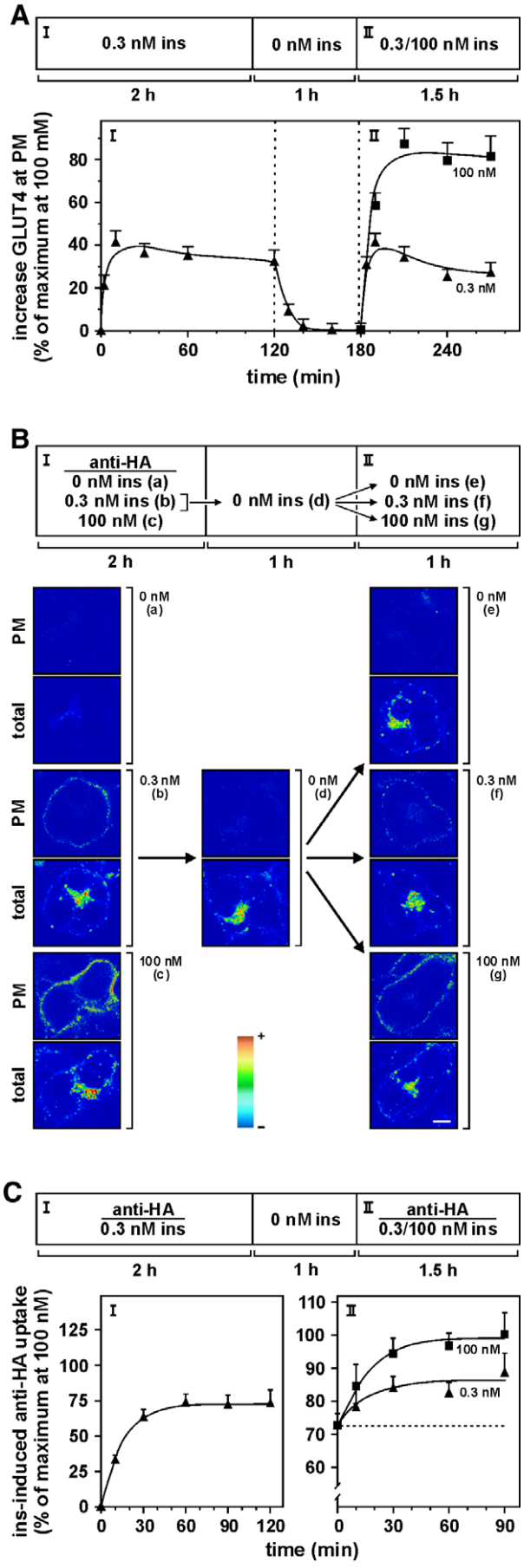
mobilized more GLUT4 molecules (Fig. 1C). As each insulin concentration provoked a distinct equilibrium, this indicated that there is hardly any mixing between mobilized and non-mobilized GLUT4 pools.

We next performed experiments to analyze the effect of insulin on (the kinetics of) the appearance of GLUT4 at the cell surface. Incubating the adipocytes with different concentrations of insulin for 20 min revealed that increasing insulin concentrations led to increases in cell surface GLUT4 levels and that maximum and half-maximum surface levels were reached at 35 nM and 1.0 nM, respectively (Fig. 2A). Moreover, analyzing cell surface GLUT4 levels in time revealed that higher insulin concentrations augmented the relative amounts of GLUT4 at the plasma membrane at steady state, in accordance with a previous study (Fig. 2B) [15]. Here, by expressing cell surface GLUT4 amounts as percentage of maximum levels for each individual insulin concentration, we show that, homologous to the situation for cycling GLUT4 levels, higher insulin concentrations did not shorten the time it took to reach final cell surface GLUT4 levels and that the kinetics of cell surface appearance was independent of insulin concentration (Fig. 2C).

Together, these data demonstrate that only a limited pool of GLUT4 participates in cell surface cycling while this pool continues to cycle as long as the insulin stimulus is present. Moreover, they show that increasing insulin concentrations result in the mobilization (i.e., cell surface recycling) of a larger pool of GLUT4 and in a concomitant increase in cell surface GLUT4 levels. Moreover, they are in line with studies that show that in adipocytes, insulin increases the amount of GLUT4-containing vesicles that are moving along microtubules rather than altering the speed of vesicle movement [16]. While there is a clear correlation between cycling and cell surface GLUT4 levels, demonstrating the importance of the graded insulin-induced release of GLUT4 into a cell surface recycling pathway, it should not be neglected however that insulin changes the GLUT4 exocytosis rate as well [15].

As the same GLUT4 molecules keep recycling under low-level insulin stimulations, it is likely that the localization of this GLUT4 pool is distinct from that of the non-mobilized pool. The non-mobilized (retained) pool and a part of the mobilized pool are probably present in distinct subcompartments of the same endosomal structures as part of the retention machinery is associated with endosomes: a GLUT4 mutant that is localized exclusively in endosomes is still retained intracellularly (though to a lesser extent) [5,11] and in fibroblasts, where GLUT4 is only present in endosomes, GLUT4 is still somewhat retained and responsive to insulin [5,17,18].

So far, these and other data support two models of insulin-induced GLUT4 translocation: one in which distinct GLUT4 subpools exist that have a distinct insulin sensitivity and one in which GLUT4 is recruited at random. According to the first model ('onion' model), insulin first releases the GLUT4 molecules that are somehow bound less tight by the retention machinery (onion outer layers). Moreover, the GLUT4 pools that are not mobilized upon a first low insulin stimulation (inner layers) will not be recruited upon subsequent low-level insulin stimulations neither and will only be mobilized when cells are stimulated with high insulin concentrations. In favour of this model are the studies that



demonstrate that even at high insulin levels, not all GLUT4 molecules engage in cell surface recycling [3,5]. The presence of such latent pool (onion core) might be the consequence of the way that the GLUT4 retention system is organized and sensitive to insulin. Such immobilizable 'silent' pools have also been described for other cell surface recycling proteins suggesting that this feature is more general than initially anticipated [19,20]. This model might in part be homologous to the situation in neuroendocrine cells where atrial natriuretic factor (ANF)-containing large dense core vesicles (LDCVs) display distinct sensitivity to stimuli and whose sensitivity depends on vesicle age [21]. In the second model, GLUT4 is recruited at random upon (low-level) insulin stimulation and GLUT4 subpools that have a distinct insulin sensitivity do not exist. According to this model, when cells are stimulated twice with a low concentration of insulin, during the second stimulus, part of the GLUT4 molecules that participated in the first stimulation are mobilized as well as molecules that were not previously mobilized.

To test which of these two models describes best how GLUT4 retention and mobilization is organized, experiments were designed in which HA–GLUT4-expressing adipocytes were stimulated with a low concentration of insulin (0.3 nM) in the presence of saturating amounts of anti-HA antibody in order to label all GLUT4 molecules that, at one point during stimulation, reached the plasma membrane. After returning to the basal (non-insulin-stimulated) state, the adipocytes were subjected to a second stimulation (0.3 nM or 100 nM) and increases in captured anti-HA antibody were determined (Fig. 3). Importantly, control experiments in which cell surface GLUT4 levels were measured demonstrated that both insulin stimulations resulted in similar cell surface GLUT4 levels and that in between these stimulations, its surface levels reduced to basal (Fig. 3A).

Moreover, to test whether the 1 h insulin washout period was sufficient for mobilized GLUT4 to return to the GLUT4 storage/exocytic compartment, an experiment was performed in which GLUT4 was labeled with anti-HA antibody during a 0.3 nM insulin treatment, followed by the 1 h insulin washout period and a subsequent 1 h incubation with or without 0.3 nM or 100 nM insulin in the absence of anti-HA antibody (Fig. 3B). Subsequently, the adipocytes were fixed, incubated with fluorescent secondary antibody to label anti-HA at the cell surface, permeabilized, and incubated with

Fig. 3. Upon consecutive low-level insulin stimulations, partially different pools of GLUT4 are mobilized. (A) Diagram displays schematic outline of experiment. HA–GLUT4-expressing adipocytes were incubated for two hours in the presence of 0.3 nM insulin. Following a mild acid wash to remove insulin, the cells were incubated with pH-neutral medium without antibody for another 50 min to allow the adipocytes to return to basal state, and then for 1.5 h in the presence of 0.3 nM or 100 nM insulin. Control cells were either incubated without insulin or with 100 nM insulin for the first 2 h (representing 100%). Cell surface GLUT4 levels were determined. (B) HA–GLUT4 was labeled with anti-HA antibody during a 0.3 nM insulin treatment, followed by a 1 h insulin washout period and a 1 h incubation in the absence (e) or presence of 0.3 nM (f) or 100 nM insulin (g). The fixed adipocytes were incubated with fluorescent secondary antibody to label anti-HA at the cell surface ('PM'), permeabilized, and incubated with another fluorescent secondary antibody to label the remaining cellular anti-HA ('total'). Control cells were fixed after the first 0.3 nM insulin treatment (b) or after the 1 h insulin washout period (d), or incubated for 2 h with antibody in the absence (a) or presence (c) of 100 nM insulin. The images are displayed in pseudo-colour to better visualize subtle differences in signal intensity. Bar in image (g) represents 5 μm. The colour bar displays the range in signal intensity from zero signal ('–') to maximum (saturated) signal ('+'). (C) Adipocytes were incubated as described under (A) except that anti-HA antibody was included during the two insulin stimulations. Control adipocytes were either incubated without insulin or with 100 nM insulin for the first 2 h (representing 100%). For the two periods of antibody uptake, the amounts of antibody captured by the adipocytes were determined and expressed as percentage of the difference between antibody captured by 0 and 100 nM insulin-treated adipocytes. The dashed line represents the assumed signal when the same GLUT4 subpools would have been recruited twice and GLUT4 would not have been recruited at random.

another fluorescent secondary antibody to label the remaining cellular anti-HA. This revealed that, in accordance with Fig. 3A, the 1 h insulin washout period was sufficient to reduce cell surface GLUT4 levels to basal (panels *b* and *d*). Moreover, these data demonstrated that previously mobilized anti-HA-bound GLUT4 was able to translocate to the cell surface upon the second stimulus (panels *f* and *g*), indicating that (part of) the GLUT4 pool that had been mobilized during the first stimulus could be remobilized during the second insulin treatment, and suggesting that the 1 h insulin washout period was likely to be sufficiently long for GLUT4 to re-enter into the GLUT4 sequestration/exocytic compartment.

Finally, experiments were performed in which live cells were exposed to anti-HA antibody during both insulin stimuli (Fig. 3C). The first 0.3 nM insulin stimulation induced an antibody uptake that was nearly half-maximal. Subsequent to the return of the cells to the basal state, the second 0.3 nM stimulation still increased the amount of antibody that was captured by the cells, indicating that GLUT4 molecules were recruited by insulin during the second stimulation that had not been recruited during the first. During this second low-level stimulation, again approximately half of the non-antibody-bound GLUT4 molecules that were maximally recruitable (i.e., with 100 nM insulin) were mobilized. These observations are novel and demonstrate for the first time that following subsequent insulin stimulations partially distinct GLUT4 pools are mobilized and thus that GLUT4 molecules are recruited at random upon insulin stimulation.

As GLUT4 molecules appear to be recruited at random it is tempting to speculate that upon low-level insulin stimulation, GLUT4 molecules are mobilized by the action of a downstream insulin effector on a protein or membrane structure that directly regulates GLUT4 retention. The result would be the release of GLUT4 molecules from the GSC into the GLUT4 cell surface recycling pathway or the release of GSVs to allow them to fuse with the plasma membrane (via the formation of the syntaxin 4/SNAP23/VAMP2 SNARE complex [22]) or perhaps with another compartment of the GLUT4 cell surface recycling pathway (i.e., via a syntaxin 13/SNAP23/VAMP2 complex [23,24]). In any case, this insulin effector would be the rate-limiting factor in GLUT4 mobilization and affect (a limited amount of) components of the retention mechanism or GSVs at a random fashion. Possibly, the tethering protein TUG or synapsin IIb could be involved [25,26]. As insulin gradually releases GLUT4 into a cell surface recycling pathway while steady states are relatively quickly reached, this means that the insulin effector is rapidly inactivated, avoiding the release of more (or all) GLUT4. Since mobilized and non-mobilized pools do not mix (distinct steady states in Fig. 1B), this also means that insulin abolishes the return of the released GLUT4 to the storage compartment. This second insulin effect is likely independent of the insulin-induced release of GLUT4 from the storage compartment and adds up to the many actions of insulin on GLUT4, including its effect on the fusion of GLUT4-containing vesicles with the plasma membrane [6] and on endosomal recycling kinetics [27,28].

Taken together, the data presented here show that upon insulin stimulation of adipocytes, GLUT4 is most probably recruited at random and mobilized from a storage compartment into a cell surface recycling pathway. The amount of GLUT4 in this cell surface recycling pathway determines at least in part the amount of GLUT4 at the plasma membrane. At this stage, these mobilized molecules do not return to or traverse the storage compartment and hence do not mix with GLUT4 molecules that were not initially mobilized by insulin. Only upon the return of the adipocytes to the basal (non-stimulated) state, almost all of the initially mobilized GLUT4 molecules leave the cell surface recycling pathway to return to the storage compartment, where they or other GLUT4 molecules can be mobilized by subsequent stimulations.

Acknowledgements

We thank Hartmut Land for pBABE vector and acknowledge the C3M MicorBio Cell Imaging Facility. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the University of Nice-Sophia-Antipolis, the Conseil Régional Provence-Alpes-Côte d'Azur, the Conseil Général des Alpes Maritimes, and an Alfediam-Roche Diagnostics award. R.G. is a recipient of an INSERM Avenir grant.

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